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In vitro methane formation and carbohydrate fermentation by rumen microbes as influenced by selected rumen ciliate species

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Abstract

Ciliate protozoa contribute to ruminal digestion and emission of the greenhouse gas methane. Individual species of ciliates co-cultured with mixed prokaryote populations were hypothesized to utilize carbohydrate types differently. In an *in vitro* batch culture experiment, 0.6 g of pure cellulose or xylan was incubated for 24 h in 40-mL cultures of *Entodinium caudatum*, *Epidinium ecaudatum*, and *Eudiplodinium maggii* with accompanying prokaryotes. Irrespective of ciliate species, gas formation (mL) and short-chain fatty acids (SCFA) concentrations (mmol L^{-1}) were higher with xylan (71; 156) than with cellulose (52; 105). Methane did not differ (7.9% of total gas). The SCFA profiles resulting from fermentation of the carbohydrates were similar before and after removing the ciliates from the mixed microbial population. However, absolute methane production (mL $24\ h^{-1}$) was lower by 50% on average after removing *E. caudatum* and *E. maggii.* Methanogen copies were less without *E. maggii*, but not without *E. ecaudatum*. Within 3 weeks part of this difference was compensated. Butyrate proportion was higher in cultures with *E. maggii* and *E. ecaudatum* than with *E. caudatum* and only when fermenting xylan. In conclusion, the three ciliate species partly differed in their response to carbohydrate type and in supporting methane formation.

Keywords: Cellulose; *Entodinium*; *Epidinium*; *Eudiplodinium*; Fiber; Hemicellulose

Introduction

Ciliate protozoa are an integral part of the rumen microbial ecosystem. Apart from degrading carbohydrates like other ruminal microbes, they specifically influence growth and activity of rumen bacteria and methanogenic Archaea. A general influence of the protozoa in this respect has been mainly explored by comparing the fermentation capacity of ruminants with intact or defaunated, *i.e.*, protozoa-free, rumen microbial populations (Eugène et al. 2004; Morgavi et al. 2008; Belanche et al. 2011a). However, defaunation-induced changes like those found for instance in ruminal short-chain fatty acid (SCFA) profile (Williams and Coleman 1992; Eugène et al. 2004; Belanche et al. 2011a) and methanogenesis (Soliva et al. 2003) were not consistent between studies. Likewise, digestibility of organic matter (OM) and fiber is not always (Kasuya et al. 2007; Zeitz et al. 2012), but often (Eugène et al. 2004; Belanche et al. 2011a) higher in faunated animals. In the latter case, hemicellulose degradation seems to be stronger inhibited by defaunation than cellulose digestion (Chaudhary et al. 1995). Further, the rumen microbes in defaunated animals may focus on easily degradable cell wall tissue and discriminate against poorly degradable cell walls whereas discrimination is less in faunated animals (Kasuya et al. 2007).

As the composition of the protozoal fauna is known to differ between diets (Hristov et al. 2001; Tymensen et al. 2012), inconsistent effects of defaunation between studies

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might result from differences in the fermentative activity of individual ciliate species. There are indications from studies performed *in vitro* (Jouany and Toillon 1997; Ranilla et al. 2007) and *in vivo* (Jouany et al. 1981) that individual ciliate species affect methane (CH_4) formation differently. However, this is not supported by the findings of Belanche et al. (2011b). Besides, the different rumen ciliate species have different constitution of genes necessary for (fiber) degradation (Bera-Maillet et al. 2005; Ricard et al. 2006) and differ in their capacity to degrade structural carbohydrates (Michałowski et al. 2001, 2003). The SCFA fermentation pattern and CH_4 formation are known to be influenced by different pure carbohydrates (Weimer 2011; Poulsen et al. 2012). However, data on the contribution of individual ciliate species to total microbial fermentation of pure carbohydrates is lacking.

In the present study, the hypotheses tested were (i) that the ruminal prokaryote population (mainly consisting of bacteria and methanogens) differs in fermentation pattern and CH_4 forming potential from different structural carbohydrates in the presence of different ruminal ciliate species, and (ii) that the ciliate species influence the ecosystem in a way that even after their removal the remaining prokaryotes continue to differ in their activity. To verify both hypotheses, three different ciliate species were isolated and cultivated *in vitro* in mixed ciliate-prokaryotic cultures. The cultures were incubated with cellulose or xylan either in the presence or absence of the respective ciliate species, and the end products of carbohydrate fermentation, protein deamination as well as CH_4 formation, and the microbial population densities, were quantified.

Material and Methods

Initial cultivation period

The selected ruminal ciliate species included *Entodinium caudatum*, which feeds mainly on starch, as well as *Epidinium ecaudatum* and *Eudiplodinium maggii*, which are amongst the ciliates with the highest fibrolytic activity (Williams and Coleman 1992). They were isolated from a sheep rumen by collecting 10–20 individuals per species, which were identified on the basis of morphological features (Zeitz et al. 2011). All cells isolated per species were transferred into 50-mL Erlenmeyer flasks sealed with a natural rubber stopper. The flasks contained 40 mL of Caudatum-type medium which is free of ruminal fluid (Coleman et al. 1972). Following the protocol of Michałowski (1995), ciliate cultures were incubated at 39 °C under exclusive CO_2 atmosphere for 2 weeks. Afterwards, cultures were propagated in medium M (Dehority 1998) for approximately 2 months before the experiment started. Medium M had well supported growth and viability of all selected species in a previous study (Zeitz et al. 2011). The media were prepared aerobically every second week, and stored at 4 °C. Ciliate protozoa were cultivated in the presence of prokaryotes in the present study. The prokaryotes had been obtained in the course of isolating the ciliates, *i.e.*, they originated either from the sheep's original rumen fluid or represented the prokaryotes which had been associated extra- or endocellularly with the picked ciliate cells. The ciliate cultures received 15 mg day^{-1} of a powdered feed composed of ryegrass hay, wheat gluten, barley flour, and crystalline cellulose (Sigmacell type 20, S3504; Sigma–Aldrich, Buchs, Switzerland) in a ratio of 0.6: 0.16: 0.12: 0.12 as described in Zeitz et al. (2011). After feeding, the culture flasks were gassed with CO_2 for 3 min at 39 °C. Cultures were transferred into fresh medium every 4 days (Zeitz et al. 2011).

Experimental design and protocol

The same source of cellulose as used during cultivation was tested as a pure carbohydrate. Both crystalline and amorphous cellulose are occurring in forages (Weimer 1992). The other carbohydrate type used was xylan (a form of highly complex polysaccharides; produced from oat spelts; product number 38500 (discontinued product; Serva, Heidelberg, Germany), serving as a model for hemicellulose. In order to standardize the carbohydrate particle size as far as possible, xylan was ground for 40 s with a ball mill (Retsch MM200, Schieritz & Hauenstein, Arlersheim, Switzerland) at 30 m s^{-1}. Additionally, xylan was sieved to exclude all particles >63 μm.

Incubations were carried out at 39 °C for 24 h in 120-mL serum bottles sealed with butyl rubber stoppers (Sigma–Aldrich, Buchs, Switzerland). Ciliate cultures were used after being allowed to grow for 4 days in fresh medium. Besides these ciliate cultures (*i.e.*, ciliates plus prokaryotes), prokaryotic cultures free of ciliates were tested. These cultures were prepared by removing the ciliates just before the incubation started through using the supernatant after centrifuging the ciliate cultures for 3 min at 2147 × *g* at ambient temperature (Kisidayova et al. 2000). From each of the ciliate-prokaryotic co-cultures ($n = 7$) and the ciliate-free cultures ($n = 4$ only, due to technical problems) 20 mL were then transferred into incubation bottles. These were filled with 20 mL of pre-warmed medium M, 0.6 g of one of the pure carbohydrates and 0.18 g of wheat gluten (G5004-500G; Sigma–Aldrich, Buchs, Switzerland) as protein source. Cultures were then gassed for 3 min with CO_2 to remove residual oxygen. Additionally, two bottles without feed served as blanks for each ciliate-prokaryotic and for each ciliate-free prokaryotic culture. The amount of gas and CH_4 produced in these blanks was subtracted from that produced by the carbohydrate-treated cultures in order to calculate net gas and CH_4 formation.

Gas production was measured after 4, 8, 20, and 24 h of incubation by using 5-mL, 10-mL and 20-mL glass syringes (Eterna Matic; Sanitex, Bassecourt, Switzerland) with disposable needles (Erosa; 23 gauge; Rose GmbH, Trier, Germany) that were plunged through the rubber stopper. The amounts of gas produced were then read from the calibrated scale of the syringe. In a preliminary test, a very high

correlation of 0.996 between gas production and gas pressure was recorded with the help of a pressure reader (GDH 200-13; Greisinger electronic, Regenstauf, Germany). After each gas withdrawal, and only then, cultures were agitated (Theodorou et al. 1994). Gas volumes were mathematically corrected to standard temperature (0 °C) and standard pressure (1013 mbar) using the ideal gas law. Additionally, prior to each gas volume measurement, 0.15 mL of gas were withdrawn from the fermentation bottle by using a gas tight syringe (Hamilton, Bonaduz, Switzerland). These samples were analyzed by gas chromatography for their CH_4 concentration (Hewlett Packard, model 5890 series II, Avondale, PA, USA). The respective amounts of gas withdrawn and gas remaining in the head-space of the bottle (63.5 mL) were used to calculate the volume of CH_4 generated. The recommended upper level of gas pressure of 480 hPa in the headspace, above which pressure and volume measured by syringes are less well correlated (Theodorou et al. 1994), was only approached in few bottles at single measurement points and when xylan was fermented. After 24 h, the bottles were put into 4 °C cold water to stop fermentation activity; corresponding data from these samples are referred to as 24-h endpoint-data in the following. In a small additional experiment, growth of ciliate-free cultures was followed for 3 weeks after the removal of ciliates from the cultures ($n = 4$ per ciliate-prokaryotic culture) also applying the 4-day media exchange schedule and offering mixed feed as described for the initial cultivation period of the ciliate stock cultures. After these 3 weeks, incubation with carbohydrates was performed and gas production and methane formation was measured again. This was done to detect if growth of free-living methanogens in ciliate-free cultures happens and therefore if changes in CH_4 formation due to adaptation of cultures to the ciliate-free state occur.

Analysis of the post-incubation medium

Amounts of 13 mL of the post-incubation medium, *i.e.* of cultures after 24 h-incubation with cellulose or xylan, were taken from every incubated culture. They were analyzed for ammonia with a potentiometer (model 713, Metrohm, Herisau, Switzerland) equipped with an ammonia-selective gas-membrane electrode (6.0506.100; Methrom, Herisau, Switzerland) and for pH with a pH meter (Calibration Check pH Meter HI223) equipped with the electrode HI 1131 (Hanna Instruments, Woonsocket, Rhode Island, USA) directly after sampling. For SCFA analysis, 4 mL of post-incubation medium were centrifuged at 2147 × *g* for 5 min and the supernatant was frozen at −20 °C until being analyzed by High Pressure Liquid Chromatography (HPLC; System Hitachi Lachrom, Merck, Tokyo, Japan) following Ehrlich et al. (1981). To count the ciliates, 0.5 mL of post-incubation medium was diluted with formaldehyde to a final formaldehyde concentration of 20 mL L^{-1}. All ciliate cells present in 0.1 mL of fixed sample were counted in duplicate on a microscope slide. Cells having disrupted pellicle or cytoplasm detaching from the cell pellicle and getting a granular structure were defined in the present study as to be 'dead', the remaining ciliates were considered as to be 'living'. Ciliate volumes were calculated from cell lengths, widths and depths measured in at least 20, 20 and 5 cells from two different experimental runs and assuming an ellipsoidal shape of ciliate cells. The correspondingly estimated ciliate volumes were 3.2, 18.4 and 34.6×10^4 μm^3 per cell for *E. caudatum*, *E. ecaudatum* and *E. maggii*, respectively. Finally, 20 µl of post-incubation medium was diluted 50-fold with Hayem solution (mmol L^{-1}: $HgCl_2$, 9; Na_2SO_4, 176; NaCl, 86), and viable prokaryotic cells showing Brownian movement were counted in duplicate within 5 h after collection with the help of a 0.02-mm depth Bürker counting chamber (Zeitz et al. 2011). From ciliate ($n = 4$) and ciliate-free ($n = 4$) cultures incubated with cellulose, extra amounts of post-incubation medium obtained after 24 h were collected for DNA extraction and its analysis of bacterial and archaeal 16S rDNA copies by real-time PCR (Zeitz et al. 2012). Calibration curves were done with PCR products of the respective primer sets described in Zeitz et al. (2012). The PCR products had been purified with the help of the illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Freiburg, Germany). The number of gene copies in the PCR reaction mix after PCR was completed was calculated from the 260 nm value, measured with a spectrophotometer (Nanodrop; Thermo Scientific, Wilmington, USA), and the size of the respective PCR product using an equation available at http://www.uri.edu/research/gsc/resources/cndna.html.

Statistical analysis

Analysis of variance was performed using the Mixed procedure of SAS (version 9.1 of 2003; SAS Institute Inc., Cary, NC). In Model 1, microbial culture (ciliate-prokaryotic culture and their corresponding ciliate-free cultures; in total six levels), time (before and after incubation) and carbohydrate type and their interactions were considered as fixed effects, and the experimental run was treated as random effect. Results of Model 1 are presented in Table 1. For the analysis of the 24 h-endpoint data by Model 2, microbial culture, carbohydrate type and the interaction were considered as fixed effects, and the experimental run was treated as random effect. Results of Model 2 are displayed in Table 2. The differences in total fermentation gas production rate and CH_4 production rate, as displayed in the figure, were analyzed under consideration of the measurements points at 4, 8, 20 and 24 h of incubation. Accordingly, Model 3 included the repeated statement. Measurement point, microbial culture and their interaction were considered as fixed effect. In Model 4, applied for the analysis of the data generated with real-time PCR, microbial culture was considered as fixed effect; the corresponding data are presented in Table 3. All multiple comparisons among means were conducted using Tukey's procedure.

Table 1. Effect of ciliate protozoa species (P) and source of carbohydrate (CH) in microbial cultures after 24 h of incubation on ciliate protozoa counts and cell volume ($n = 7$ per mean displayed).

Trait/CH	Before incubation			After 24 h of incubation			SE	*P*-values			
	EC	*EE*	*EM*	*EC*	*EE*	*EM*		P	CH	Time	P × CH × time
Ciliates, living cells (mL^{-1})							279.7	<0.001	0.64	<0.001	0.69
Cellulose	4782^{b}	242^{c}	319^{c}	7589^{a}	271^{c}	296^{c}					
Xylan				7083^{a}	251^{c}	397^{c}					
Ciliates, dead cells, % of living cells							1.480	<0.001	0.85	<0.001	0.74
Cellulose	0.94^{c}	4.37^{bc}	2.26^{c}	1.16^{c}	5.02^{bc}	12.39^{a}					
Xylan				1.48^{c}	5.87^{abc}	10.25^{ab}					
Ciliates, living cells cell volume ($mm^3\ mL^{-1}$)							0.0113	<0.001	0.66	<0.001	0.17
Cellulose	0.155^{b}	0.045^{d}	0.110^{bc}	0.246^{a}	0.050^{d}	0.102^{c}					
Xylan				0.229^{a}	0.046^{d}	0.137^{bc}					

[a–d] Means within trait with different superscripts are different at $P<0.05$.

Results

Effect of ciliate species and carbohydrate type

Before setting up the 24-h fermentation experiments and across all microbial cultures ($n = 42$), prokaryotic counts were $1.1 \pm 0.36 \times 10^9\ mL^{-1}$, pH was 7.3 ± 0.11, and ammonia concentration was $4.2 \pm 0.71\ mmol\ L^{-1}$ (data not shown). There was no significant difference between microbial cultures in these variables.

In the post-incubation medium, *i.e.* in cultures after 24 h-incubation with cellulose or xylan, the counts of living ciliates differed between ciliate-containing cultures, but were similar between carbohydrates (Table 1). During 24 h, living cell counts in *E. caudatum* cultures increased compared to the initial counts, but cell counts of *E. ecaudatum* and *E. maggii* remained similar (Table 1). Only in the *E. maggii* cultures, the percentage of dead cells increased compared to the initial counts both when incubated with cellulose and xylan. The volume of living ciliates was the largest in cultures with *E. caudatum*, intermediate in the case of *E. maggii* and the smallest volume characterized *E. ecaudatum*. However, the relative differences in cell volumes were much lower compared to those in counts. Counts of prokaryotes were higher in cultures supplied with xylan than with crystalline cellulose after 24 h of incubation, but within carbohydrate, they were similar in all microbial cultures (Table 2). Bacterial 16S rDNA copies, analyzed in cultures incubated with cellulose, were similar in all microbial cultures as well (Table 3).

During incubation, incubation medium pH decreased and reached 5.8 (cellulose) and 5.4 (xylan) after 24 h of incubation, but was not influenced by the ciliate species present in the ciliate-prokaryotic cultures. In cultures supplemented with cellulose, but not in those supplemented with xylan, the post-incubation medium pH in ciliate-prokaryote cultures was lower than in ciliate-free cultures, but was similar in the three ciliate-free cultures (Table 2). The ammonia concentration in post-incubation medium was similar across all microbial cultures, but higher with cellulose compared to xylan. When comparing the three ciliate-prokaryote cultures, the concentration of total SCFA did not depend on the presence of the ciliate species, but it was higher when xylan was fermented, compared to cellulose. In cultures where ciliates had been removed, the SCFA concentrations were similar compared to the ciliate-containing cultures, and did not differ between carbohydrates either. The SCFA profile did not differ between microbial cultures and carbohydrates except for butyrate. In xylan-fermenting ciliate-prokaryote cultures, *E. caudatum* cultures had a lower butyrate proportion and a higher butyrate: propionate ratio than those of *E. ecaudatum* ($P < 0.01$ and $P < 0.001$, respectively) and *E. maggii* ($P = 0.14$ and $P < 0.001$, respectively). These differences were still partly found in the ciliate-free cultures. Accordingly, the butyrate-to-propionate ratio was also lower in *E. caudatum*-free cultures than in *E. ecaudatum*- ($P < 0.05$) and *E. maggii*-free cultures ($P < 0.001$). However, the proportion of butyrate did not differ between ciliate-free cultures.

Net formation of fermentation gases after 24 h of incubation was influenced by both carbohydrate and microbial culture (Table 2). Although gas volume was similar within ciliate-prokaryotic and within ciliate-free cultures for each carbohydrate, it was influenced by the presence of *E. caudatum*. In *E. caudatum*-containing cultures, the volume of gas produced was higher than in its respective ciliate-free culture, but not different in cultures supplemented with cellulose and xylan. In the cultures containing *E. ecaudatum* and *E. maggii*, gas volume was higher when supplemented with xylan than with cellulose but similar in ciliate-prokaryotic and ciliate-free cultures. Although the gas production rates were similar when comparing the microbial cultures containing either *E. caudatum*, *E. ecaudatum* or *E. maggi*, the gas production kinetics were influenced by the presence of the ciliates as such because gas production rates after 8 h were sometimes lower in ciliate-free cultures. During the last 4 h of the incubation period (*i.e.*, 20–24 h), differences between gas production rates between ciliate-prokaryote and

Table 2. Effect of ciliate protozoa species (P) and source of carbohydrate (CH) in microbial cultures after 24 h of incubation in the presence and absence of the examined species of ciliates on prokaryote counts and various traits after 24 h of incubation ($n = 7$ runs for the ciliate-prokaryote culture; $n = 4$ for the ciliate-free culture).

Trait/CH	Ciliate-prokaryote culture			Ciliate-free culture			SE	*P*-values		
	EC	*EE*	*EM*	*EC*	*EE*	*EM*		P	CH	P × CH
Prokaryotes ($\times 10^9$ mL^{-1})							0.405	0.32	<0.001	0.075
Cellulose	3.29^{b}	3.28^{b}	3.47^{b}	2.60^{b}	2.41^{b}	2.94^{b}				
Xylan	5.56^{a}	6.64^{a}	6.53^{a}	5.86^{a}	7.28^{a}	6.60^{a}				
pH							0.039	<0.001	<0.001	<0.001
Cellulose	5.76^{b}	5.76^{b}	5.76^{b}	6.05^{a}	6.09^{a}	5.97^{a}				
Xylan	5.48^{c}	5.45^{c}	5.46^{c}	5.44^{c}	5.47^{c}	5.44^{c}				
Ammonia (mmol L^{-1})							1.019	0.32	<0.001	0.19
Cellulose	11.43^{a}	9.83^{abc}	9.54^{abc}	11.29^{ab}	10.70^{abc}	8.48^{abcd}				
Xylan	6.34^{bcd}	4.44^{d}	6.48^{bcd}	4.32^{d}	3.46^{d}	5.52^{cd}				
Short-chain fatty acids (mmol L^{-1})							12.12	0.48	<0.001	0.53
Cellulose	113.8^{ab}	106.1^{b}	95.4^{b}	92.2^{b}	108.8^{ab}	109.8^{ab}				
Xylan	153.6^{a}	154.3^{a}	159.3^{a}	128.0^{ab}	137.4^{ab}	138.0^{ab}				
Acetate (%)							2.82	0.21	0.11	0.87
Cellulose	75.5	78.3	78.8	79.1	79.5	86.0				
Xylan	75.0	72.9	78.5	77.3	75.4	80.1				
Propionate (%)							2.08	0.047	0.19	0.63
Cellulose	18.4	16.2	16.1	14.4	15.9	9.2				
Xylan	19.6	17.5	13.3	18.3	18.4	13.6				
Butyrate (%)							0.811	0.038	0.0071	0.0054
Cellulose	4.51^{b}	4.06^{b}	3.80^{b}	4.70^{ab}	3.41^{b}	3.49^{b}				
Xylan	3.96^{b}	8.65^{a}	7.30^{ab}	2.89^{b}	5.00^{ab}	5.18^{ab}				
Isobutyrate (%)							0.180	0.022	0.84	0.54
Cellulose	0.56	0.50	0.50	0.93	0.46	0.58				
Xylan	0.83	0.39	0.42	0.81	0.56	0.61				
Valerate (%)							0.132	0.97	0.16	1.00
Cellulose	0.51	0.58	0.48	0.42	0.50	0.37				
Xylan	0.36	0.38	0.31	0.37	0.36	0.32				
Isovalerate (%)							0.090	0.69	0.0058	0.81
Cellulose	0.55	0.39	0.37	0.50	0.33	0.40				
Xylan	0.24	0.22	0.19	0.32	0.32	0.17				
Propionate: butyrate							0.469	<0.001	0.31	<0.001
Cellulose	4.76^{ab}	4.20^{b}	4.47^{ab}	3.23^{bc}	4.86^{ab}	3.03^{bc}				
Xylan	5.03^{ab}	2.05^{c}	2.05^{c}	6.85^{a}	3.89^{bc}	2.93^{bc}				
Net gas (mL 24 h^{-1})							3.51	<0.001	<0.001	0.24
Cellulose	54.4^{bcd}	51.7^{cd}	50.0^{cde}	34.2^{e}	44.1^{de}	43.0^{de}				
Xylan	66.5^{ab}	70.3^{a}	76.3^{a}	50.0^{cde}	62.2^{abc}	64.2^{abc}				
Net CH_4 (mL 24 h^{-1})							0.603	<0.001	<0.001	<0.001
Cellulose	4.24^{bc}	3.78^{bcde}	3.97^{bcd}	2.27^{de}	3.38^{bcde}	3.06^{cde}				
Xylan	4.98^{b}	4.79^{bc}	7.39^{a}	1.94^{e}	4.75^{bc}	3.48^{bcde}				
Net CH_4 (mL 100 mL^{-1} gas)							1.305	0.17	0.30	0.082
Cellulose	7.79	7.16	7.90	8.65	7.04	6.42				
Xylan	7.39	7.58	9.60	3.22	7.74	5.21				

[a–e] Means within trait with different superscripts are different at $P < 0.05$.
EC = *Entodinium caudatum*, EE = *Epidinium ecaudatum*, EM = *Eudiplodinium maggii*. Net gas: Gas produced in blanks without substrate was subtracted from the gas produced by the cultures which received the carbohydrates.

ciliate-free cultures had mostly disappeared or the relationship had even been reversed, suggesting that fermentation was setting off slower without the ciliates (Fig. 1A, B).

Net CH_4 volume was only influenced by the type of microbial culture when xylan was fermented, but not with cellulose (Table 2). With xylan, in the *E. maggi*-containing culture the volume of CH_4 produced within 24 h was about 50% higher than in cultures containing *E. caudatum* and *E. ecaudatum*. The removal of the three different ciliate species affected CH_4 production rates and the volume of CH_4 produced in the ciliate-free cultures differently. Compared to their respective ciliate-containing cultures, only cultures where *E. caudatum*

Table 3. Effect of ciliate species in microbial cultures incubated without or with prior removal of the ciliates on copies of Bacteria and Archaea, and on methane formation related to archaeal copies or protozoal cells after 24 h of incubation with cellulose ($n = 4$).

Trait/species	Ciliate-prokaryote culture	Ciliate-free culture	SE	*P*-value
Bacterial copies (10^{10} mL^{-1})			0.277	0.29
E. caudatum	2.01	2.09		
E. ecaudatum	2.54	2.43		
E. maggii	2.37	2.91		
Archaeal copies (10^{8} mL^{-1})			0.452	0.006
E. caudatum	3.14^{ab}	2.03^{b}		
E. ecaudatum	4.17^{a}	3.81^{ab}		
E. maggii	4.18^{a}	2.17^{b}		
Archaeal copies related to protozoal cells (10^{6} copies protozoal cell^{-1})			0.47	0.076
E. caudatum	0.13			
E. ecaudatum	2.01			
E. maggii	1.43			
Net CH_4 related to archaeal copies (μmol CH_4 10^{8} copies^{-1})			0.109	0.0004
E. caudatum	1.26^{a}	0.82^{bc}		
E. ecaudatum	0.69^{c}	0.70^{c}		
E. maggii	0.87^{bc}	1.09^{ab}		
Net CH_4 related to protozoal cells (μmol CH_4 10^{3} protozoal cells^{-1})			3.01	0.023
E. caudatum	0.6^{b}			
E. ecaudatum	14.3^{a}			
E. maggii	12.6^{a}			
Net CH_4 related to protozoal volume (μmol CH_4 mm^{-3} protozoal volume)			15.38	0.075
E. caudatum	18.9			
E. ecaudatum	75.0			
E. maggii	44.0			

[a–c] Means within trait with different superscripts are different at $P < 0.05$.

and *E. maggi*, but not those where *E. ecaudatum* had been removed, produced less CH_4 (Table 2) and had a lower CH_4 production rate (Fig. 1B) in the early incubation hours. The CH_4 proportion of total gas did not differ (Table 2). Archaeal 16S rDNA copies, analyzed in cultures growing on cellulose, differed between the *E. maggii*-containing and the *E. maggii*-free culture ($P < 0.05$), but not between ciliate-containing and ciliate-free cultures of *E. caudatum* and *E. ecaudatum* ($P > 0.1$) (Table 3). The methane volume produced during 24 h was related to archaeal copies determined after 24 h (μmol CH_4 per 10^{8} archaeal copies) in cultures supplemented with cellulose. This showed that CH_4 production per methanogen cell was higher in *E. caudatum*-containing than in *E. caudatum*-free culture and was also higher than in cultures containing *E. ecaudatum* and *E. maggii*. In contrast, CH_4 production per protozoal cell was the lowest in cultures containing *E. caudatum*; however, CH_4 production per protozoal cell volume was similar in all cultures (Table 3).

Within 3 weeks of cultivation of the ciliate-free cultures (additional experiment, see materials and methods), CH_4 formation had increased as compared to the levels measured directly after preparation to 4.1 ± 1.9, 3.3 ± 1.2 and 2.2 ± 1.3 fold levels in the cases of the ciliate-free cultures prepared from *E. caudatum*, *E. maggii* and *E. ecaudatum*, respectively (data not shown). When CH_4 formation from cellulose and xylan in these 3-week old ciliate-free cultures was related to that of the ciliate-prokaryotic cultures 3 weeks before, they produced 72 ± 6, 111 ± 16, and 105 ± 7% of the former amounts in cases of *E. caudatum*, *E. maggii* and *E. ecaudatum*, respectively.

Discussion

Effects of carbohydrate type

The lower post-incubation medium pH, together with higher gas and SCFA formation in cultures supplemented with xylan as found in the present study, illustrates a higher degradability of this carbohydrate compared to the cellulose. A higher gas production from fermented pure xylan (hemicellulose) as compared to pure crystalline cellulose found in the present study has been reported earlier by Fuente et al. (2009) when diluted rumen fluid was incubated. Crystalline cellulose has also been found being degraded by enzyme preparations from microbial cultures with *E. ecaudatum* at a lower rate than xylan and carboxymethylcellulose (Michałowski et al. 2001). However, in the latter study, this did not seem to be the result of ciliate metabolism. The higher degradation of xylan in comparison to cellulose in the present study was associated with higher prokaryotic counts whereas ciliate counts were unaffected by the carbohydrate type. This illustrates

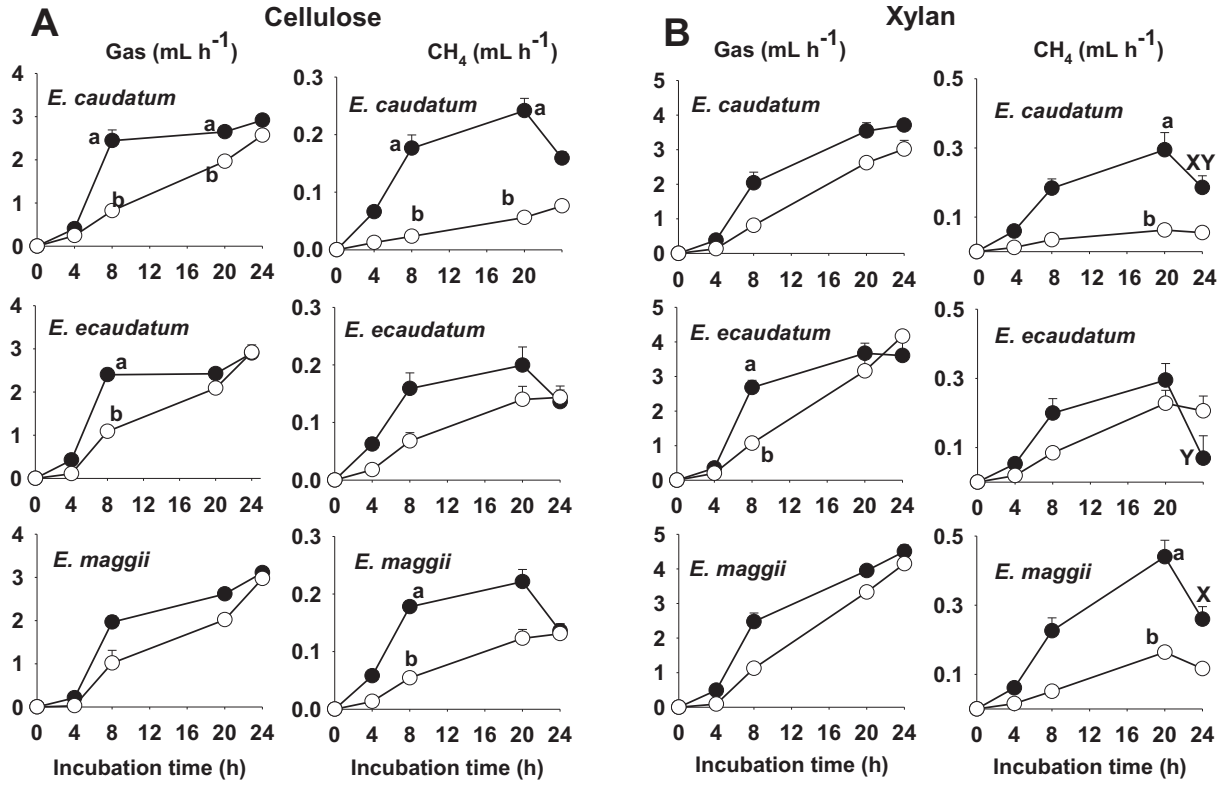

Fig. 1. Production rates of total fermentation gas (right) and methane (left), as calculated using the gas volume produced between two measurement points, from *in vitro* incubation of cellulose (A) and xylan (B) with the ciliate cultures *E. caudatum*, *E. ecaudatum* and *E. maggii* and their corresponding ciliate-free cultures. —●— ciliate-prokaryotic culture, —○— ciliate-free culture. Bars represent standard errors. For each time point, unequal capital letters (X,Y) denote differences ($P < 0.05$) between ciliate cultures in subgraphs at $P < 0.05$, and unequal lowercase letters (a,b) denote differences ($P < 0.05$) between the ciliate-prokaryotic culture and the corresponding ciliate-free culture.

the importance of prokaryotic fermentation in the *in vitro* cultures where substrate amounts supplied had been high. The assumption that prokaryotic fermentation was important is consistent with the observation of a higher gas production with xylan occurring in both the ciliate-prokaryotic and the ciliate-free cultures. Changes in the SCFA profile are mainly expected when structural and non-structural carbohydrates are compared but not between cellulose and xylan which are both structural carbohydrates. Consistent with this expectation, the SCFA profile was hardly influenced by the carbohydrate type in the present study.

Whereas structural carbohydrates generally lead to higher CH_4 formation than *e.g.* starch, a non-structural carbohydrate (Kreuzer et al. 1986), findings are inconsistent concerning the difference between cellulose and hemicelluloses. Equations to predict enteric CH_4 generally assume that a higher volume of CH_4 is produced per kg of digested cellulose than per kg of digested hemicellulose (Hindrichsen et al. 2005). However, *in vivo* CH_4 formation is not only influenced by the nutrients entering the rumen but also by the different passage rate of the feeds – a factor which is kept constant in batch cultures. Therefore limiting the incubation time may artificially limit gas and CH_4 production from slowly degradable carbohydrates like cellulose compared to faster degradable carbohydrates like xylan. This may mask differences between cellulose and xylan degradation. It has been reported that the degradation of hemicelluloses leads to a higher CH_4 formation per unit of dry matter supplied as compared to cellulose when batch-incubated in rumen fluid *in vitro*; however, this difference was no longer evident after 123 transfers and 9.7 months of cultivation (Weimer 2011). In the present study, 2.5 months after isolation of the microbial cultures from the rumen, also no difference between cellulose and xylan in terms of CH_4 production (mL $24\,h^{-1}$ and % of total gas) has been found (exception: higher CH_4 volume with xylan in the *E. maggii*-containing culture).

Effects of ciliate species and their corresponding ciliate-free cultures

In the present study, the ciliate concentration in the pre- and post-incubation medium was much lower than *in vivo* (Zeitz et al. 2012), which is, however, often the case in ciliate cultures (Kisidayova et al. 2000). Therefore, the influence of the ciliates on microbial fermentation in the present study was probably lower than under conditions which more closely resemble *in vivo* conditions in terms of ciliate

concentration. This might be one explanation for the observation that the presence of ciliates in the microbial cultures did not clearly enhance the intensity of fermentation compared to the cultures of ruminal prokaryotes alone. Different from that, Kisidayova et al. (2000), using the same ciliate removal technique and with similar ciliate concentrations in the cultures, described that the absence of the ciliates in former *in vitro* ciliate cultures may lead to a clearly lower gas formation. Furthermore, it has to be stated that, due to the isolation procedure used for the prokaryote cultures in the present study, it could not be avoided that certain large bacteria were also removed and thus were absent in the supernatant used as ciliate-free prokaryote culture. Therefore, the prokaryote population in the ciliate-free cultures probably differed from that of the ciliate-containing cultures. However, similar gas and total SCFA production by both ciliate-prokaroytic and ciliate-free cultures might also illustrate the presence of a strong competition for feed between ruminal ciliates and prokaryotes, with the latter being able to replace ciliates in fermentation *in vitro* within few hours. It is known that ciliates can control fermentation by the rapid ingestion of feed particles like entire starch granules, thus impeding bacterial accession to feed (Kisidayova et al. 2000), and store them for eventual degradation. This shall help prevent a fast decline in ruminal pH by slowing down fermentation. Unexpectedly, the fermentation of all nutrients was more rapid and intensive in the presence of the ciliates whereas gas and CH_4 production in the ciliate-free cultures started later but increased steadily and reached the same production rates at the end of the 24-h fermentation period. The rapid fermentation of the ciliate-containing cultures might be partly explained by their property to remove oxygen from rumen fluid thereby creating suitable conditions for more susceptible anaerobic bacteria (Ellis et al. 1989), and additionally, by the high amounts of feed supplied in the present study resulting in presumed unhampered prokaryotic fermentation also in the presence of ciliates.

In the present study, butyrate proportion of total SCFA (6%) was low in all cultures compared to other *in vivo* (Michałowski et al. 2003) and *in vitro* (Ranilla et al. 2007) studies, although still in a range measured in the rumen environment (Yáñez-Ruiz et al. 2007; Owens et al. 2009). This was unexpected for the ciliate cultures because butyrate is an important end product of ciliate metabolism (Gutierrez and Davies 1962; Williams and Coleman 1992) and thus, when ciliates are present, frequently higher molar proportions of butyrate are found as compared to defaunated animals (Eugène et al. 2004; Yáñez-Ruiz et al. 2007). However, the present finding was probably related to the low ciliate concentration. Despite these limitations, it was shown that, in cultures growing on xylan, the presence of single ciliate species within a mixed microbial culture influenced butyrate proportion (*E. caudatum* < *E. ecaudatum*) and butyrate-to-propionate ratio (*E. caudatum* > *E. ecaudatum* = *E. maggii*). With a mixed prokaryote-ciliate inoculum mimicking rumen ciliate concentrations, butyrate proportions were higher in the presence of *Entodinium* sp. as compared to *e.g. Eudiplodinium in vitro* and were higher than in the absence of protozoa, but only in the inoculum containing *Entodinium* sp. (Ranilla et al. 2007). In the same study, concentrations of *Entodinium* were much higher as compared to the other ciliates, and a high correlation ($r = 0.9$) between ciliate cell volume and butyrate had been found. In contrast, in the present study butyrate concentrations in cultures with *E. caudatum* were lowest and butyrate proportion did not decrease after ciliate removal. However, even though *E. maggii* has been shown to enhance ruminal butyrate production, the establishment of *Entodinium* as second species added to an established *E. maggii* population even decreased either butyrate concentration or production rate (Michałowski et al. 2003). This emphasizes the importance of at least some ciliate species in determining the SCFA profile, especially when ciliate biomass is sufficiently high.

Defaunation has been found to decrease CH_4 emissions *in vivo* (Hegarty et al. 2008), even over a period of up to 2 years (Morgavi et al. 2008), but also to remain without effect (Soliva et al. 2003; Hegarty et al. 2008). In the present study, the removal of ciliates from microbial cultures shortly before starting the incubation with carbohydrates partly decreased CH_4 production. However, it seems that the extent to which CH_4 production decreases is dependent on ciliate species and on how much time was left after ciliate removal before measurement. The lack of a general relationship is illustrated by the absence of a significant correlation between CH_4 production and ciliate biomass (Ranilla et al. 2007). Inconsistent effects of defaunation further emphasize that factors other than the presence of ciliates as such might be decisive for ruminal CH_4 formation (Soliva et al. 2003). The influence of the ciliates on CH_4 formation might indeed be species-specific. For instance, *E. caudatum* had been found to increase *in vitro* CH_4 formation as compared to *Isotricha* and *Metadinium* species (Ranilla et al. 2007). In the present study, the removal of *E. ecaudatum* did not influence either CH_4 formation or archaeal 16S rDNA copies, whereas both were decreased by the removal of *E. caudatum*, at least when cellulose was fermented. The association of the methanogens with the three investigated ciliate species is known to differ. A proportion of 40–55% of *E. caudatum* and *E. maggii* cells have been found to be associated with methanogens, but only 20% of the *E. ecaudatum* cells (Vogels et al. 1980). Consistent with this, the removal or addition of *E. caudatum* and, sometimes, *E. maggii* from or to a defaunated rumen (Takenaka and Itabashi 1995; Zeitz et al. 2012), ruminal fluid (Ranilla et al. 2007) or ciliate cultures (Kisidayova et al. 2000) was associated with concomitant changes in either CH_4 production or methanogen counts or both. However, substrates varied within and between these studies making general conclusions sometimes difficult. The present study demonstrated that the baseline level of CH_4 production, which was initially lower after removal of *E. maggii* and *E. caudatum*, was partially or totally restored within 3 weeks. This indicates that,

depending on the ciliate species removed, in defaunated animals a certain compensatory growth of the methanogens may take place. Besides a possible influence of diet type (Kreuzer et al. 1986; Hegarty et al. 2008), this might be one possible explanation for controversial findings concerning the influence of defaunation on CH_4 emissions.

Differences in fermentation of pure carbohydrates by the ciliate species studied

In the present study, functionally clearly different ciliate species and carbohydrate types have been investigated. Two of the species are optionally fibrolytic with high cellulolytic (Williams and Coleman 1992) and xylanolytic (Williams and Coleman 1992; Michałowski et al. 2001) activity. Different from that, the third species (*E. caudatum*) is non-cellulolytic and mainly relying on starch or non-polysaccharide carbohydrates (Williams and Coleman 1992). Consequently, it had been expected that effects of ciliate species co-cultured with a mixed ruminal prokaryotic population occur when fermenting the structural carbohydrates supplied. In the case of cellulose fermentation, net CH_4 volume and production rate were similar in all microbial cultures, whereas they differed when xylan was fermented. The reason why CH_4 formation from xylan was only higher in cultures with *E. maggii*, but not with the other fibrolytic species, *E. ecaudatum*, as compared to *E. caudatum* might be the difference of association of *E. maggii* and *E. ecaudatum* with the methanogens (Vogels et al. 1980). Furthermore, *E. caudatum* might influence the cellulolytic prokaryote population in a way that cellulose degradation and CH_4 formation are supported to the same extent than in cultures with *E. maggii*. Gas and SCFA production was not differently influenced by the three ciliate species examined. This suggests that fibrolytic prokaryote activities are promoted in the presence of the non-fibrolytic *E. caudatum.* This has been shown for instance in sheep faunated exclusively with *E. maggii* or with both *E. maggii* and *E. caudatum* (Michałowski et al. 2003). In sheep fed a diet rich in cellulose, *E. caudatum* as the only species present in the rumen enhanced fiber digestibility nearly to the same extent as *Polyplastron multivesiculatum* when compared to defaunated sheep (Jouany and Sénaud 1979). As CH_4 production per methanogenic cell was enhanced in cultures with *E. caudatum*, this ciliate may also stimulate activity of methanogens as shown by the cultures supplemented with cellulose in the present study. Again cultures with the two fibrolytic ciliate species caused butyrate proportion to get higher than with *E. caudatum* only when fermenting xylan, but not with cellulose. It seems that the fibrolytic ciliates contributed less to cellulose fermentation than to the degradation of xylan. In microbial cultures with *E. ecaudatum*, xylan degradation has been faster than that of crystalline cellulose, too; however, the contribution of the ciliates themselves might have been actually lower in cultures supplied with xylan (Michałowski et al. 2001). Alternatively, the prokaryotic fermentation pathways might have been influenced in a way that butyrate production was supported.

It seems that, concerning the expression of typical ciliate properties like the formation of butyrate and their influence on CH_4 formation, single ciliate species indeed influence fermentation differently, but this influence is modified by the dominant carbohydrate type available.

Conclusion

The findings of the present study partially supported the initially presented hypotheses. It became obvious that the ruminal prokaryotic population has a different fermentation pattern and CH_4 forming potential from different carbohydrate types in the presence of different ruminal ciliate species (hypothesis (i)). The fibrolytic species *E. ecaudatum* and *E. maggii* were especially important as butyrate producers. The presence of *E. caudatum* and *E. maggii*, but not of *E. ecaudatum*, had a CH_4 enhancing effect. Hypothesis (ii) that ciliate species can influence the ecosystem in a way that the remaining prokaryotes differ in their activity even after their removal could not be answered satisfactorily. Further studies have to show if ciliate-specific effects also occur with mixed diets *in vitro* and *in vivo*.

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References

Belanche, A., Abecia, L., Holtrop, G., Gnada, J.A., Castrillo, C., de la Fuente, G., Balcells, J., 2011a. Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chyme. J. Anim. Sci. 89, 4163–4174.

Belanche, A., de la Fuente, G., Doran, S.E., Moorby, J.M., Newbold, C.J., 2011b. Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation and methane emissions. In: Proceedings of the 8th International Symposium on the Nutrition of Herbivores, Aberyswyth, Wales, UK.

Bera-Maillet, C., Devillard, E., Cezette, M., Jouany, J.-P., Forano, E., 2005. Xylanases and carboxymethylcellulases of the rumen protozoa *Polyplastron multivesiculatum*, *Eudiplodinium maggii* and *Entodinium* sp. FEMS Microbiol. Lett. 244, 149–156.

Chaudhary, L.C., Srivastava, A., Singh, K.K., 1995. Rumen fermentation pattern and digestion of structural carbohydrates in buffalo (*Bubalus bubalis*) calves as affected by ciliate protozoa. Anim. Feed Sci. Technol. 56, 111–117.

Coleman, G.S., Davies, J.I., Cash, M.A., 1972. The cultivation of the rumen ciliates *Epidinium ecaudatum* and *Polyplastron multivesiculatum in vitro*. J. Gen. Microbiol. 73, 509–521.

Dehority, B.A., 1998. Generation times of *Epidinium caudatum* and *Entodinium caudatum*, determined *in vitro* by transferring at various time intervals. J. Anim. Sci. 76, 1189–1196.

Ehrlich, G.G., Goerlitz, D.F., Bourell, J.H., Eisen, G.V., Godsy, E.M., 1981. Liquid-chromatographic procedure for fermentation product analysis in the identification of anaerobic bacteria. Appl. Environ. Microbiol. 42, 878–885.

Ellis, J.E., Williams, A.G., Lloyd, D., 1989. Oxygen consumption by ruminal microorganisms: protozoal and bacterial contributions. Appl. Environ. Microbiol. 55, 2583–2587.

Eugène, M., Archimède, H., Sauvant, D., 2004. Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. Livest. Prod. Sci. 85, 81–97.

Fuente, G. de la, Belanche, A., Fondevila, M., 2009. *In vitro* ruminal fermentation of carbohydrates by Spanish ibex. Arch. Zootec. 58, 357–362.

Gutierrez, J., Davies, E., 1962. Culture and metabolism of the rumen ciliate *Epidinium ecaudatum* Crawley. Appl. Microbiol. 10, 305–308.

Hegarty, R.S., Bird, S.H., Vanselow, B.A., Woodgate, R., 2008. Effects of the absence of protozoa from birth or from weaning on the growth and methane production of lambs. Br. J. Nutr. 100, 1220–1227.

Hindrichsen, I.K., Wettstein, H.-R., Machmüller, A., Jörg, B., Kreuzer, M., 2005. Effect of the carbohydrate composition of feed concentrates on methane emission from dairy cows and their slurry. Environ. Monit. Assess. 107, 329–350.

Hristov, A.N., Ivan, M., Rode, L.M., McAllister, T.A., 2001. Fermentation characteristics and ruminal ciliate protozoal populations in cattle fed medium- or high-concentrate barley-based diets. J. Anim. Sci. 79, 515–524.

Jouany, J.-P., Sénaud, J., 1979. Role of rumen protozoa in the digestion of food cellulosic materials. Ann. Rech. Vét. 10, 261–263.

Jouany, J.-P., Toillon, S., 1997. Effect of addition of *Isotricha* spp. or a mixed fauna to defaunated rumen contents on methane production measured *in vitro*. Reprod. Nutr. Dev. 37, 44–45.

Jouany, J.-P., Zainab, B., Sénaud, J., Groliere, C.A., Grain, J., Thivend, P., 1981. Rôle of the rumen ciliate protozoa *Polyplastron multivesiculatum*, *Entodinium* sp. and *Isotricha prostoma* in the digestion of a mixed diet in sheep. Reprod. Nutr. Dev. 21, 871–884.

Kasuya, N., Wada, I., Shimada, M., Kawai, H., Itabashi, H., 2007. Effect of presence of rumen protozoa on degradation of cell wall constituents in gastrointestinal tract of cattle. Anim. Sci. J. 78, 275–280.

Kisidayova, S., Varadyova, Z., Zelenak, I., Siroka, P., 2000. Methanogenesis in rumen ciliate cultures of *Entodinium caudatum* and *Epidinium ecaudatum* after long-term cultivation in a chemically defined medium. Folia Microbiol. 45, 269–274.

Kreuzer, M., Kirchgessner, M., Müller, H.L., 1986. Effect of defaunation on the loss of energy in wethers fed different quantities of cellulose and normal or steamflaked maize starch. Anim. Feed Sci. Technol. 16, 233–241.

Michałowski, T., 1995. The influence of cellulose, starch and different sources of nitrogen on the growth of rumen ciliate protozoon *Epidinium ecaudatum in vitro*. J. Anim. Feed Sci. 4, 255–261.

Michałowski, T., Bełzecki, G., Kwiatkowska, E., Pajak, J.J., 2003. The effect of selected rumen fauna on fibrolytic enzyme activities, bacterial mass, fibre disappearance and fermentation pattern in sheep. J. Anim. Feed Sci. 12, 45–64.

Michałowski, T., Rybicka, K., Wereszka, K., Kasperowicz, A., 2001. Ability of the rumen ciliate *Epidinium ecaudatum* to digest and use crystalline cellulose and xylan for *in vitro* growth. Acta Protozool. 40, 203–210.

Morgavi, D.P., Jouany, J.-P., Martin, C., 2008. Changes in methane emission and rumen fermentation parameters induced by refaunation in sheep. Aust. J. Exp. Agric. 48, 69–72.

Owens, D., McGee, M., Boland, T., O'Kiely, P., 2009. Rumen fermentation, microbial protein synthesis, and nutrient flow to the omasum in cattle offered corn silage, grass silage, or whole-crop wheat. J. Anim. Sci. 87, 658–668.

Poulsen, M., Jensen, B.B., Engberg, R.M., 2012. The effect of pectin, corn and wheat starch, inulin and pH on *in vitro* production of methane, short chain fatty acids and on the microbial community composition in rumen fluid. Anaerobe 18, 83–90.

Ranilla, M.J., Jouany, J.-P., Morgavi, D.P., 2007. Methane production and substrate degradation by rumen microbial communities containing single protozoal species *in vitro*. Lett. Appl. Microbiol. 45, 675–680.

Ricard, G., McEwan, N.R., Dutilh, B.E., Jouany, J.-P., Macheboeuf, D., Mitsumori, M., McIntosh, F.M., Michałowski, T., Nagamine, T., Nelson, N., Newbold, C.J., Nsabimana, E., Takenaka, A., Thomas, N.A., Ushida, K., Hackstein, H.P., Huynen, M.A., 2006. Horizontal gene transfer from bacteria to rumen ciliates indicates adaptation to their anaerobic, carbohydrates-rich environment. BMC Genomics 7, 22–34.

Soliva, C.R., Hess, H.-D., Meile, L., Kreuzer, M., Machmüller, A., 2003. Suppression of ruminal methanogenesis by dietary means: apparent inconsistency between methane release and counts of microbes involved in methanogenesis. Trop. Subtrop. Agroecosyst. 3, 209–213.

Takenaka, A., Itabashi, H., 1995. Changes in the population of some functional groups of rumen bacteria by changing the rumen ciliates in calves. J. Gen. Appl. Microbiol. 41, 377–387.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B., France, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48, 185–197.

Tymensen, L., Barkley, C., McAllister, T.A., 2012. Relative diversity and community structure analysis of rumen protozoa according to T-RFLP and microscopic methods. J. Microbiol. Met. 88, 1–6.

Vogels, G.D., Hoppe, W.F., Stumm, C.K., 1980. Association of methanogenic bacteria with rumen ciliates. Appl. Environ. Microbiol. 40, 608–612.

Weimer, P.J., 1992. Cellulose degradation by ruminal microorganisms. Crit. Rev. Biotechnol. 12, 189–223.

Weimer, P.J., 2011. End product yields from the extraruminal fermentation of various polysaccharide, protein and nucleic acid components of biofuels feedstocks. Biores. Technol. 102, 3254–3259.

Williams, A.G., Coleman, G.S., 1992. The Rumen Protozoa. Springer-Verlag, New York.

Yáñez-Ruiz, D.R., Williams, S., Newbold, C.J., 2007. The effect of absence of protozoa on rumen biohydrogenation and the fatty acid composition of lamb muscle. Br. J. Nutr. 97, 938–948.

Zeitz, J.O., Amelchanka, S.L., Michałowski, T., Wereszka, K., Meile, L., Hartnack, S., Kreuzer, M., Soliva, C.R., 2012. Effect of the rumen ciliates *Entodinium caudatum*, *Epidinium ecaudatum* and *Eudiplodinium maggii*, and combinations thereof, on ruminal fermentation and total tract digestion in sheep. Arch. Anim. Nutr. 66, 180–199.

Zeitz, J.O., Meile, L., Michałowski, T., Kreuzer, M., Soliva, C.R., 2011. Suitability of different media for *in vitro* cultivation of the ruminal protozoa species *Entodinium caudatum*, *Eudiplodinium maggii*, and *Epidinium ecaudatum*. Eur. J. Protistol. 47, 314–317.